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(6 AND (TRANSPOSABLE ADJ ELEMENT)).USPT,JPAB,EPAB,DWPI.	0

Database: US Patents Full-Text Database; US Pre-Grant Publication Full-Text Database; JPO Abstracts Database; EPO Abstracts Database; Derwent World Patents Index; IBM Technical Disclosure Bulletins

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ADAPTER$1	0
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☐ 1. Document ID: US 6235503 B1

L6: Entry 1 of 4 File: USPT May 22, 2001

US-PAT-NO: 6235503
DOCUMENT-IDENTIFIER: US 6235503 B1

TITLE: Procedure for subtractive hybridization and difference analysis

DATE-ISSUED: May 22, 2001

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Lindemann; Garrett W.	Benicia	CA	N/A	N/A
Schueler; Paula A.	Benicia	CA	N/A	N/A

US-CL-CURRENT: 435/91.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

☐ 2. Document ID: US 6051376 A

L6: Entry 2 of 4 File: USPT Apr 18, 2000

US-PAT-NO: 6051376
DOCUMENT-IDENTIFIER: US 6051376 A

TITLE: Uses of mda-6

DATE-ISSUED: April 18, 2000

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Fisher; Paul B.	Scarsdale	NY	N/A	N/A
Jiang; Hongping	New York	NY	N/A	N/A

US-CL-CURRENT: 435/6; 435/69.1, 436/501, 514/2, 514/44

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

☐ 3. Document ID: US 5958738 A

L6: Entry 3 of 4 File: USPT Sep 28, 1999

US-PAT-NO: 5958738
DOCUMENT-IDENTIFIER: US 5958738 A

TITLE: Procedure for subtractive hybridization and difference analysis

DATE-ISSUED: September 28, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Lindemann; Garrett W.	Benicia	CA	N/A	N/A
Schueler; Paula A.	Benicia	CA	N/A	N/A

US-CL-CURRENT: 435/91.2

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

☐ 4. Document ID: US 5643761 A

L6: Entry 4 of 4 File: USPT Jul 1, 1997

US-PAT-NO: 5643761
DOCUMENT-IDENTIFIER: US 5643761 A

TITLE: Method for generating a subtracted cDNA library and uses of the generated library

DATE-ISSUED: July 1, 1997

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Fisher; Paul B.	Scarsdale	NY	N/A	N/A
Jiang; Hongping	New York	NY	N/A	N/A

US-CL-CURRENT: 435/91.1; 435/488, 435/489, 435/6, 435/69.1, 435/810, 435/91.2, 436/501, 536/23.1, 536/24.1, 536/24.3, 536/24.31, 536/24.32, 536/24.33

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image

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L6: Entry 3 of 4 File: USPT Sep 28, 1999

DOCUMENT-IDENTIFIER: US 5958738 A
TITLE: Procedure for subtractive hybridization and difference analysis

ABPL:
Improved methods for obtaining polynucleotides comprising sequences which differ between two populations of DNA or cDNA are provided. Improvements include reduction in the number of amplification cycles, use of a nuclease digestion step prior to amplification, a novel oligonucleotide adapter for the practice of the improved method, and novel methods for selective amplification of the desired unique fragments and selective degradation of fragments containing sequences common to both populations. Fragments of a sample population are amplified using a primer that endows the amplification products with resistance to nuclease degradation. Fragments of a control population are amplified using a primer that targets the amplification products for preferential degradation. Multiple cycles of hybridization, nuclease treatment and amplification are utilized to provide enrichment of fragments unique to the sample population. Such unique fragments may represent new or amplified sequences in the sample population, sequences that are differently arranged in the sample population compared to the control population, and sequences that are differentially expressed in a cDNA population. A variation of the technique also allows the isolation of fragments representing deletions in the sample population.

BSPR:
The invention is in the field of genetic analysis. The invention relates to methods for isolation of polynucleotides comprising nucleic acid sequences which are differentially expressed, differentially present, or differentially arranged in two or more different cells, cell populations or cell types, utilizing techniques of subtractive hybridization and selective amplification.

BSPR:
Strauss and Ausubel, Proc. Natl. Acad. Sci. USA 87:1889-1893 (1990) described a technique for isolating a polynucleotide comprising DNA that is absent in a yeast deletion mutant. In this method, denatured wild-type DNA is allowed to anneal with biotin-labeled DNA from the deletion mutant, and biotin-containing duplexes (which contain sequences common to the mutant and wild-type) are removed from solution by binding to avidin-coated beads. The process is repeated for several cycles, with addition of fresh biotinylated wild-type DNA to the mutant DNA remaining unbound at the end of each cycle. Finally, single-stranded material is amplified by a polymerase chain reaction to generate a probe enriched in sequences missing in the deletion mutant. Of course, this method can only be used to isolate a genomic region that is defined by a deletion mutant, and its applicability to genomes more complicated than that of yeast has not been tested. A similar procedure using biotin-based separation for isolation of differentially expressed cDNAs was described by Lebeau et al., Nucleic Acids Research 19: 4778 (1991).

BSPR:
Recently, a technique known as Representational Difference Analysis (RDA) has been developed, which allows the isolation of DNA fragments that are present in one population of DNA sequences but absent in another population of DNA sequences. Lisitsyn et al., Science 259:946-951 (1993); Lisitsyn et al., Meth. Enzymology 254:291-304 (1995); U.S. Pat. No. 5,436,142; U.S. Pat. No. 5,501,964; Lisitsyn et al., Nature Genetics 6:57-63 (1994). This method allows one to search for fragments present in a "tester" population of DNA sequences that are not present in a related "driver" population. Such unique fragments are denoted "target" sequences. In the first step of RDA, "representations" of both

populations are obtained. These representations consist of lower-complexity subsets of the original sequence populations. In the most widely-practiced embodiment of the technique, a representation is obtained by separately subjecting both populations to digestion with a restriction endonuclease, ligating a first set of adapters to the ends of the fragments so generated, and amplifying by a polymerase chain reaction (PCR) using primers complementary to the first set of adapters, under conditions in which only relatively short fragments (less than 2 kilobase pairs) are amplified. The first adapters are then removed from the amplified fragments of both populations by restriction enzyme digestion and a second set of adapters (having a different sequence than the first set) is attached, by ligation, to amplified fragments from the tester DNA population only.

BSPR:
The adapter-containing amplified fragments from the tester population are then combined with an excess of amplified fragments from the driver population, (which lack adapters) and the mixture is incubated under denaturing and annealing conditions, followed by another round of PCR amplification using primers complementary to the second set of adapters. During the annealing step, several types of duplex will be formed. Because driver fragments are present in excess, the vast majority of fragments containing sequences common to both tester and driver populations will form either driver-driver duplexes (containing no adapter) or tester-driver duplexes (containing a single adapter on the strand derived from the tester fragment). Fragments containing sequences that are unique to the tester population are capable of self-annealing to generate duplexes possessing an adapter at each end. Consequently, during the PCR step subsequent to annealing, tester-tester duplexes will be amplified exponentially. On the other hand, tester-driver duplexes, possessing only a single adapter, will be amplified in a linear fashion and will thus come to form only a small fraction of the population of amplified sequences. Driver:driver duplexes, lacking adapters, will not be amplified at all. Selective amplification of fragments containing target sequences is thus achieved by virtue of the fact that, prior to annealing, only fragments from the tester population possess adapters, bestowing tester-tester duplexes with the potential for exponential amplification.

BSPR:
The steps of removing the adapters present on the enriched target fragments obtained from a previous step, adding new adapters, incubating under denaturing and annealing conditions with an excess of fragments from the driver population, and amplification by PCR is repeated until a desired degree of enrichment is attained.

BSPR:
As the technique of RDA has come to be practiced more widely in recent years, several disadvantages have become apparent. A major problem results from the inefficiency of the multiple restriction digestion and ligation reactions that are utilized in the technique. Lack of complete restriction digestion will lead to incomplete removal of the first set of adapters from the tester fragment population, resulting in an inability to attach the second set of adapters. Similarly, an inefficient ligation step would lead to incomplete attachment of the second set of adapters, even at sites from which the first set had been removed. Since the amplification primers are complementary to the second set of adapters, incomplete attachment of the second adapter set will reduce the degree of amplification of target sequences that can be achieved. In addition, the necessity to process samples through multiple steps, and possibly purify material between steps, leads to losses of already-scarce experimental material. One possible consequence of inefficient restriction digestion and/or ligation is the generation of false positives, wherein the loss of a particular driver sequence, through failure to be amplified, leads to the inappropriate identification of its complement in the tester as a target sequence.

BSPR:
Another disadvantage of RDA as it is commonly practiced stems from the use of a large number of polymerase chain reaction cycles during the amplification step. Typically, 20 cycles of PCR are used to generate the representations and 25-30 cycles of PCR are used during each hybridization/amplification round of RDA. If, as is common, three rounds of hybridization/amplification are conducted, target nucleic acids will have undergone 95-110 rounds of amplification by the time they are isolated. Additional rounds of amplification are commonly used to clone and

sequence the difference product isolated by RDA. It has been known for some time that, at high cycle numbers of a PCR amplification, a "plateau effect" is observed. Innis and Gelfland in "PCR Protocols: A guide to methods and applications" ed. Innis et al., Academic Press (1990) pp. 3-12. This effect is characterized by a decline in the exponential rate of accumulation of amplification product that occurs during late cycles. Potential causes of the plateau effect include 1) depletion of substrates, 2) loss of activity of enzyme, 3) degradation of substrates, 4) end-product inhibition, 5) competition for reactants by nonspecific products, 6) incomplete denaturation of product at high product concentration and 7) reannealing of product at high product concentration (which may block primer annealing and/or extension).

BSPR:
These last two features of the later cycles of a polymerase chain reaction are especially important for RDA and related techniques because, besides leading to less-than-exponential amplification, they also result in a skewing of the representation of products in reactions, such as RDA, in which multiple fragments are being amplified. In particular, Mathieu-Daude et al., Nucleic Acids Research 24:2080-2086 (1996) have shown that, in later cycles, the rate of amplification of abundant products decreases more rapidly than that of less abundant products in the same reaction. This is due to preferential reannealing of the more abundant products, which prevents primer binding and/or extension for these abundant species. This phenomenon is consistent with the fact that rate of annealing is proportional to the concentrations of the reacting strands. The consequence of this effect for the practice of cDNA-RDA is that the ability to detect mRNAs present in different concentrations in two populations (as opposed to mRNAs that are unique to one of the populations) will be minimized for mRNAs whose cDNAs are present at high concentrations in the starting population.

BSPR:
Finally, the presence of excess driver DNA during the ten PCR cycles prior to nuclease treatment can result in a reduced efficiency of amplification of tester:tester hybrids, due to the potential for the residual driver:driver and driver:tester duplexes to act as a sink for primers, substrates, counterions and enzyme.

BSPR:
In the practice of the present invention, these disadvantages are surmounted by methods that use fewer PCR cycles, nuclease digestion prior to amplification, and a single adapter designed for use with multiple primers. Additional advantages are also presented by the invention, as set forth infra.

BSPR:
The invention also provides a method for obtaining additional difference products by repeating the preceding series of steps (b), wherein the first difference product (or a subsequent difference product) is substituted for the lower-complexity subset of the sample fragment population, and wherein the treated annealing mixture is subjected to amplification using a non-targeting primer complementary to an internal primer binding site different from that used in the preceding step or a non-targeting primer complementary to the innermost primer binding site.

BSPR:
b) covalently attaching to both fragment populations an oligonucleotide containing or encoding nested primer binding sites, said primer binding sites comprising an outermost primer binding site, an innermost primer binding site, and at least one more internal primer binding site therebetween, to produce oligonucleotide-labeled sample and control fragment populations;

BSPR:
c) amplifying the oligonucleotide-labeled sample fragment population, using a non-targeting primer complementary to the outermost primer binding site of said oligonucleotide to generate an amplified sample fragment population;

BSPR:
d) amplifying the oligonucleotide-labeled control fragment population, using a targeting primer complementary to the innermost primer binding site of said oligonucleotide to generate an amplified control fragment population;

BSPR:
f) subjecting the annealing mixture to conditions under which all fragments will be degraded, except for double-stranded fragments containing a non-targeting primer in each strand; and

BSPR:
g) subsequently subjecting the annealing mixture treated as in step (f) to amplification, using non-targeting primers complementary to one of said internal primer binding sites, to generate a first difference product.

BSPR:
The invention also provides a method for obtaining additional difference products by repeating the preceding series of steps (e) through (g), wherein the first difference product (or a subsequent difference product) is substituted for the amplified sample fragment population, and wherein the treated annealing mixture is subjected to amplification using a non-targeting primer complementary to an internal primer binding site different from that used in the preceding step or a non-targeting primer complementary to the innermost primer binding site, to provide additional difference products.

BSPR:
b) covalently attaching to both fragment populations an oligonucleotide comprising nested primer binding sites or the complements thereof, said primer binding sites comprising an outermost primer binding site, an innermost primer binding site, and at least one more internal primer binding site therebetween, to produce marked sample and control fragment populations;

BSPR:
c) amplifying the marked sample fragment population using a non-targeting primer complementary to the outermost primer binding site of said oligonucleotide to generate an amplified sample fragment population; and

BSPR:
d) amplifying the marked control fragment population using a targeting primer complementary to the innermost primer binding site of said oligonucleotide to generate an amplified control fragment population; and

BSPR:
The invention also provides a method for obtaining additional difference products by repeating the preceding series of steps (e), wherein the first difference product (or a subsequent difference product) is substituted for the amplified sample fragment population, and wherein the treated annealing mixture is subjected to amplification using a non-targeting primer complementary to an internal primer binding site different from that used in the preceding step or a non-targeting primer complementary to the innermost primer binding site.

BSPR:
In another embodiment, an oligonucleotide having multiple primer binding sites for use in the method of the invention and related methods is provided.

BSPR:
In another embodiment, an oligonucleotide having multiple primer binding sites with increasing annealing temperatures from the 5'- to the 3'-end, for use in the method of the invention and related methods, is provided.

BSPV:
(ii) covalently attaching to both fragment populations an oligonucleotide comprising nested primer binding sites, said primer binding sites comprising an outermost primer binding site, an innermost primer binding site, and at least one more internal primer binding site therebetween, to produce marked sample and control fragment populations;

BSPV:
(iii) amplifying the marked sample fragment population under conditions wherein the degree of amplification is limited, using a non-targeting primer complementary to the outermost primer binding site of said oligonucleotide to generate a lower-complexity subset of the sample fragment population; and

BSPV:

(iv) amplifying the marked control fragment population under conditions wherein the degree of amplification is limited, using a targeting primer complementary to the innermost primer binding site of said oligonucleotide to generate a lower-complexity subset of the control fragment population; and

BSPV:
(ii) subjecting the annealing mixture to conditions under which all fragments will be degraded, except for double-stranded fragments containing a non-targeting primer in each strand; and

BSPV:
(iii) subsequently subjecting the annealing mixture to amplification, using non-targeting primers complementary to one of said internal primer binding sites, to generate a first difference product.

BSPW:
(ii) subjecting the annealing mixture to conditions under which all fragments will be degraded, except for double-stranded fragments containing a non-targeting primer in each strand;

BSPW:
(iii) subsequently subjecting the annealing mixture to amplification, using non-targeting primers complementary to one of said internal primer binding sites, to generate a first difference product.

DEPR:
As used herein, marking refers to a method by which additional sequences are added to a polynucleotide or nucleic acid fragment. A marked population of polynucleotide fragments may be generated, for example, by the attachment of oligonucleotide linkers or adapters to a population of polynucleotide fragments. Differential marking refers to a situation in which the presence of the additional sequences is exploited to distinguish two or more populations of polynucleotides or polynucleotide fragments from one another.

DEPR:
Amplification is the process by which additional copies of a nucleic acid sequence or collection of nucleic acid sequences are generated. Amplification is generally achieved enzymatically, using a DNA polymerase enzyme. Current techniques allow exponential amplification of any sequence flanked by binding sites for a pair of oligonucleotide primers, through reiterative application of denaturation, primer annealing and polymerase extension steps, commonly known as a polymerase chain reaction. U.S. Pat. No. 4,683,202, Saiki et al., Science 239:487-491 (1988), Innis et al., supra, Erlich, supra. Under the most widely-practiced conditions of the polymerase chain reaction, the rate of polymerization is approximately 1,000-2,000 nucleotides per minute. Accordingly, the maximum length of amplifiable sequence will be limited by the reaction conditions (for example, the duration of the extension step). The ability to control the extent of elongation in a polymerase chain reaction can be used to advantage to generate lower-complexity subsets of amplified fragments from an initial fragment collection of high complexity.

DEPR:
A primer is an oligonucleotide capable of base-pairing with a polynucleotide and serving as a site from which polymerization can be initiated.

DEPR:
A primer having properties such that its extension product will be susceptible to degradation is known as a targeting primer; a primer having properties such that its extension product is protected from degradation is known as a non-targeting primer. More generally, a targeted population of polynucleotides is one that is preferentially susceptible to degradation by virtue of some unique property or constituent that is not shared with a non-targeted population of polynucleotides.

DEPR:
A primer binding site refers to a region of a polynucleotide, such as an adapter or a sequence encoded by an adapter, that is capable of base-pairing with a primer, or that encodes a sequence that is able to base-pair with a primer. By way of example, the adapters of the present invention may contain within their

sequence or encode multiple primer binding sites. In the case of multiple primer binding sites, the outermost primer binding site is the primer binding site located closest to the 3' end of the nucleic acid strand in which it resides. The innermost primer binding site is located farthest from the 3' end of the nucleic acid strand in which it resides. One or more internal primer binding site(s) may be present between the outermost and innermost primer binding sites. It should be noted that, since polymerization proceeds in a 5'-to-3' direction and a primer binding site is complementary to the primer from which polymerization is initiated, an outermost primer binding site encodes a sequence closest to the 5'-end of the product of polymerization, compared to internal or innermost primer binding sites.

DEPR:
Certain nucleic acid populations, such as those representing the mRNA population of a cell, may be of sufficiently low complexity that the generation of a lower-complexity subset may not be necessary. In this case, digestion with a restriction enzyme having a four-nucleotide recognition sequence will facilitate the subsequent attachment of adapters to provide an amplified fragment population for use in the annealing step and lead to amplification of virtually all fragments (since the majority of fragments produced by cleavage with a restriction enzyme having a four-nucleotide recognition sequence will be shorter than 2 kilobases).

DEPR:
C. Adapter oligonucleotides

DEPR:
The present invention provides novel adapter oligonucleotides, whose use leads to greater efficiency and higher yields of authentic difference product, by obviating the necessity for repeated replacement of adapters by restriction enzyme digestion and ligation. These novel adapter oligonucleotides contain or encode binding sites for several individual primers, which may be used for fragment amplification and, if desired, to target certain of the resultant amplified fragments for selective degradation. The novel oligonucleotides are of sufficient length so as to contain several primer binding sites, but not so long as to interfere with the specificity of hybridization (i.e., by containing regions of self-complementarity). The multiple primer binding sites contained within or encoded by an adapter may overlap one another to generate nested primer binding sites, or the primer binding sites may be discrete.

DEPR:
In a preferred embodiment, each sequence encoding a primer binding site, progressing from the 5' end to the 3' end of the adapter will have a successively higher annealing temperature. The difference in annealing temperature between adjacent sites will be approximately 10.degree. C., more preferably 5.degree. C. and, most preferably, 3.degree. C. Consequently, the guanine+cytosine content and/or the length of each primer binding site will also increase, in progressing from the 5' end to the 3' end of the adapter. Thus, in general, the 5'-most, or outermost primer binding site will be shorter, have a lower guanine+cytosine content, and a lower annealing temperature, while the 3'-most, or innermost primer binding site will be longer, have a higher guanine+cytosine content, and a higher annealing temperature. The range of annealing temperatures for the individual primer binding sites of the adapter should, at the low end, be sufficiently high to allow specific hybridization and, at the high end, not be inhibitory to the DNA polymerase used for amplification. This range will be from about 20.degree. C. to 90.degree. C., more preferably from about 35.degree. C. to about 85.degree. C., still more preferably between about 45.degree. C. to about 80.degree. C. and most preferably from about 55.degree. C. to about 75.degree. C. In a preferred embodiment, the oligonucleotide adapter contains four primer binding sites. However, it is clear that more or less than four primer binding sites would also be encompassed by the invention, and that there need not necessarily be any specific relationship among the annealing temperatures of the primer binding sites.

DEPR:
The adapters are attached to fragments by techniques that are well-known in the art. Adapters may be attached chemically or enzymatically, via the action of a DNA or RNA ligase (e.g., see Maniatis et al., Sambrook et al., Ausubel et al., supra). In a preferred method for attachment of adapters, fragmentation of a DNA

population will be achieved by treatment with a restriction enzyme that leaves a 5'-protruding end. A short oligonucleotide, part of which is complementary to this 5'-overhang and part of which is complementary to the sequence at the 3'-end of the adapter is used to align the adapter for attachment of the 3'-terminus of the adapter to the 5'-terminus of the restriction enzyme-generated end. After attachment of the adapter, the short oligonucleotide is removed by denaturation and the complement of the adapter is synthesized by the action of a DNA polymerase, using the 3' end of the original fragment as a primer.

DEPR:
If lower-complexity subsets of the control and sample polynucleotide populations are to be generated by selective amplification of small fragments, adapters are attached to the members of the population prior to this step; and it is preferred that the sample fragment population is amplified using a non-phosphorylated primer complementary to the outermost primer binding site, while the control fragment population is amplified using a phosphorylated primer complementary to the innermost primer binding site.

DEPR:
After attachment of adapters, the control and sample fragment populations are separately subjected to an initial amplification step, preferably by a polymerase chain reaction. Generation of the complement of the adapter sequence, as just described, can be accomplished prior to the initial amplification by conducting an initial extension in the absence of primer.

DEPR:
Through the process of this initial amplification step, the amplification products of the sample and control fragment populations will become differentially marked. This differential marking will target the amplified fragments of the control population for selective degradation, and insure that non-targeted duplexes (arising during subsequent annealing steps) from the amplified fragments of the sample population are resistant to degradation. Differential marking is accomplished by using different types of primers for amplification of the control fragment population and the sample fragment population, as will now be described.

DEPR:
Primers capable of annealing to the primer binding sites of the oligonucleotide adapter may be either phosphorylated or non-phosphorylated at their 5' end. It is within the skill of the art to prepare either type of primer by automated synthesis (see, e.g., Applied Biosystems Model 380D DNA synthesizer User's Manual and associated technical reports, Gait, supra, and Eckstein, supra, the disclosures of which are hereby incorporated by reference). Phosphorylated oligonucleotides are synthesized on an automated instrument by using a phosphorylated monomer in the final coupling cycle. Such a phosphorylated monomer for use in automated synthesis may be obtained commercially, for example from Clontech Laboratories, Palo Alto, Calif. or Applied Biosystems, Foster City, Calif. or Glen Research, Sterling, Va. or other commercial vendors. Alternatively, a phosphorylated oligonucleotide may be prepared through automated synthesis of a 5'-hydroxyl-terminated oligonucleotide, followed by enzymatic phosphorylation using methods well-known in the art (e.g. Maniatis et al., Sambrook et al., Ausubel et al., supra).

DEPR:
In a preferred embodiment of the present invention, phosphorylated and non-phosphorylated primers will be used in the differential marking of two populations of polynucleotide fragments, as follows. In the initial amplification of the control fragment population, a 5'-phosphorylated primer is used for amplification. In the initial amplification of the sample population, a 5'-non-phosphorylated primer is used for amplification. The presence of a 5'-phosphate group on fragments comprising the amplified control fragment population renders those fragments susceptible to the action of nucleases which initiate exonucleolytic degradation at 5'-phosphate-termini, such as .lambda. exonuclease. By contrast, lack of a 5'-phosphate on fragments comprising the sample fragment population protects these fragments from the action of 5'-phosphate-specific nucleases such as X exonuclease. A primer whose extension products are susceptible to degradation is known as a targeting primer and its extension products are said to be targeted; while a primer whose extension products are protected from degradation is known as a non-targeting primer and

its extension products are said to be non-targeted. Other types of non-targeting primer include those containing one or more a-phosphorothioate or methyl phosphonate internucleotide linkages near the 5' end, or primers whose 5'-end is blocked with amine or thiol groups, all of which also yield extension products that are resistant to degradation by .lambda. exonuclease. Other types of 5' end modification [i.e., modified nucleic acid structures such as, for example, bicyclo DNA, Bolli et al. (1996) Nucleic acids Res. 24: 4660-4667, and peptide nucleic acids, Nielsen et al. (1991) Science 254:1497-1500] which render an oligo- or polynucleotide resistant to a 5'-specific exonuclease are also contemplated by the invention. In addition, other types of nuclease can be used for degradation of targeted nucleic acid strands. For example, T7 gene 6 exonuclease is a double strand-specific exonuclease that hydrolyzes in a 5'-to-3' direction. Kerr and Sadowski (1989) J. Biol. Chem. 247:311-318. However, digestion by T7 gene 6 exonuclease can be blocked by the presence of four or more phosphorothioate bonds at the 5' end of a double-stranded DNA molecule. Nikiforov et al. (1994) PCR Meth. & App. 3:285-291. Monomers and reagents useful for the incorporation of amino- or thio-modified nucleotides during automated oligonucleotide synthesis are available from various commercial suppliers, for example Clontech Laboratories, Palo Alto, Calif. or Applied Biosystems, Foster City, Calif. or Glen Research, Sterling, Va., or other suppliers.

DEPR:
For the production of non-targeted amplification products, amplification is primed with a 5' hydroxyl-terminated primer complementary to a primer binding site different from the one used for amplification of the targeted fragment population. Generally, the amplified control fragment population will be targeted and the amplified sample fragment population will be non-targeted.

DEPR:
In the most preferred embodiment of the initial amplification step of the invention, the control fragment population will be amplified using a phosphorylated primer having a sequence complementary to the innermost primer binding site of the adapter, and the sample fragment population will be amplified using a non-phosphorylated primer having a sequence complementary to the outermost primer binding site of the adapter.

DEPR:
The ionic strength of an annealing mixture is traditionally adjusted using the Na.sup.+ cation. However, the Mg.sup.2+ cation would be preferable for several reasons. First, a given concentration of Mg.sup.2+ will provide correspondingly higher ionic strength, compared to an equal concentration of a monovalent cation such as Na.sup.+ or K.sup.+. Second, the high concentration of Na.sup.+ used in annealing reactions (typically approximately 1 M) is inhibitory to the DNA polymerase enzymes used for amplification, and is too high to allow dilution of the annealing mixture into the amplification reaction while maintaining reasonable reaction volumes, necessitating precipitation or purification of the annealed product prior to amplification. Heretofore, the use of Mg.sup.2+ in annealing reactions was precluded by the frequent presence of Mg.sup.2+ dependent nucleases which often contaminated preparations of DNA and RNA. In the present invention, nucleic acids to be amplified have generally been subjected to a sufficient number of purification steps (e.g., before and after ligation of adapters) that nuclease contamination is negligible. Alternatively, nucleic acid preparations can be purified by chromatography on specially-designed matrices, such as Qiaex II (Qiagen) or GeneClean (Bio 101) to yield nuclease-free preparations. Consequently, the present invention contemplates the use of Mg.sup.2+ in the annealing reaction at a concentration of 62.5 mM, which is equivalent to 1 M NaCl in terms of ionic strength. Wetmur and Sninsky in "PCR Strategies" ed. Innis et al., Academic Press (1995). pp.69-83. After the annealing reaction is complete, the reaction mixture may simply be diluted into the components of the amplification reaction such that the Mg.sup.2+ concentration is between 1-5 mM, depending upon the Mg.sup.2+ optimum of the DNA polymerase used for amplification, which is generally about 1.5 mM.

DEPR:
Accordingly, the annealing step is followed by one or more nuclease digestion steps, in which targeted polynucleotides are susceptible to degradation. In a preferred embodiment, targeting primers contain a 5'-phosphate group and non-targeting primers are non-phosphorylated at their 5'-ends; and a combination of Mung Bean Nuclease and .lambda. exonuclease is used for digestion. These

conditions will result in the destruction of single-stranded material by Mung Bean Nuclease, and the exonucleolytic degradation (by .lambda. exonuclease) of any strand terminated with a 5'-phosphate group. Thus, duplexes containing both strands from the control fragment population will be degraded. Duplexes containing one 5'-phosphorylated strand (from the control fragment population) and one strand that is non-phosphorylated at the 5'-end (from the sample fragment population) will undergo degradation of the 5'-phosphorylated strand by .lambda. exonuclease, after which the remaining strand becomes susceptible to Mung Bean Nuclease action. Duplexes consisting of two strands from the amplified sample fragment population will contain two 5'-hydroxyl termini and are thus preferentially resistant to degradation. Such duplexes are also preferentially amplifiable, as will be described below. Digestions with these enzymes may proceed in either order; in a preferred embodiment, an initial digestion with .lambda. exonuclease is followed by treatment with Mung Bean Nuclease. Enzymes or chemical treatments which have the same specificity as Mung Bean Nuclease and .lambda. exonuclease are also contemplated by the present invention.

DEPR:
Following nuclease treatment, the surviving undigested polynucleotides are amplified using a non-targeting primer complementary to one of the internal primer binding sites, preferably the first internal primer binding site adjacent to the outermost primer binding site. As described supra, duplexes containing sequences unique to the sample population will have survived nuclease degradation by virtue of their non-phosphorylated 5'-ends. They will thus be available for exponential amplification. All other species of duplex or single stranded polynucleotide will have been degraded by the preceding nuclease treatment. Consequently, the amplification product will be highly enriched for fragments that are unique to the sample population.

DEPR:
The amplified material obtained from the preceding step may be combined with an excess (2-fold to 100,000-fold, preferably 100-fold) of amplified fragments from the control population, and the cycle of annealing, nuclease digestion and amplification may be repeated until one or more desired difference products are obtained. At each cycle, a different non-targeting primer is used for the amplification step. In a preferred embodiment, the amplification primer at any given cycle will correspond to the next primer binding site internal to that used in the previous cycle. A non-targeting primer complementary to the innermost primer binding site may be used, if necessary.

DEPR:
Double stranded cDNA is prepared using an oligo 5'-T.sub.30 MN-3' primer (M=A, G or C and N=A, G, C, or T), following the double-stranded cDNA production protocol that accompanies the Gibco BRL (Gaithersburg, Md.) Superscript.TM. II RT enzyme. Preparations of cDNA are made from both the sample and control RNA populations, under identical conditions in separate reaction vessels.

DEPR:
D. Ligation of Adapters

DEPR:
4.3 .mu.l desalted Linker Adapter 13-mer oligo LA(1 mg/ml) [SEQ ID NO:2]

DEPR:
2 .mu.l Primer P1 (1 mg/ml) [SEQ ID NO:3]

DEPR:
2 .mu.l Adapter-ligated sample fragment population, diluted as in D above

DEPR:
2 .mu.l primer PP4 (1 mg/ml) [SEQ ID NO:7]

DEPR:
2 .mu.l Adapter-ligated control fragment population, diluted as in D above

DEPR:
The reactions are placed in a PTC 100.TM. thermocycler (M J Research, Watertown, Mass.) with Hot Bonnet and incubated for 3 minutes at 80.degree. C. (to dissociate the Linker Adapter 13-mers from step D). One microliter (5 Units)

Vent.RTM. (exo) DNA polymerase (New England Biolabs, Beverly, Mass.) is added and incubation at 80.degree. C. is continued for 5 minutes. This fills in the ends complementary to the 45-mer adapter oligonucleotide that was ligated to the fragments in step D, thereby generating a set of primer binding sites. Next, the reaction mixtures are subjected to twenty cycles of 1 minute at 95.degree. C. and 3 minutes at 74.9.degree. C. A final extension of 10 minutes at 75.degree. C. is performed, then the reactions are cooled to 4.degree. C. It is important not to increase the cycle number beyond 20, as a skewing toward smaller amplification products will occur, which will bias the subsequent subtraction. If more product is required, the number of reactions should be increased.

DEPR:
To prepare an annealing mixture, 80 .mu.l of the amplified, adapter-ligated control fragment population from step E above (40 .mu.g), is mixed with 40 .mu.l of a 50-fold dilution of the amplified, adapter-ligated sample fragment population from step E above (0.4 .mu.g), in a 0.5 ml Eppendorf microfuge tube. The mixture is extracted once with phenol/chloroform and once with chloroform. Nucleic acids are precipitated by addition of 30 .mu.l of 10 M NH.sub.4 OAc, 380 .mu.l 100% EtOH and incubation at -70.degree. C. for 10 minutes. The mixture is warmed to 37.degree. C. for 1 to 2 minutes (to minimize salt precipitation) and then subjected to centrifugation at 14,000 rpm for 14 minutes at 4.degree. C. The pellet is washed twice with 85% EtOH, with brief centrifugation each time, then dried by vacuum for three minutes.

DEPR:
2 .mu.l P2 primer (1 mg/ml) [SEQ ID NO:4]

DEPR:
DP-1 is diluted to 10 ng/.mu.l with TE. The steps of subtractive hybridization (F), dilution (G. 1), Mung Bean Nuclease Digestion (G.2), .lambda. Exonuclease digestion (G.3), and amplification (G.4) are repeated, following the procedures described above, with the following changes. In step F, DP-1 is substituted for the amplified, adapter-ligated sample fragment population; in step G.4, the P3 primer [SEQ ID NO: 5] is substituted for the P2 primer [SEQ ID NO: 4] and the following cycling program is used for amplification using the P3 primer: 1 minute at 95.degree. C. and 3 minutes at 72.1.degree. C.

DEPR:
DP-2 is diluted to 10 ng/.mu.l with TE. The steps of subtractive hybridization (F), dilution (G. 1), Mung Bean Nuclease Digestion (G.2), .lambda. Exonuclease digestion (G.3), and amplification (G.4) are repeated, following the procedures described above, with the following changes. In step F, DP-2 is substituted for the amplified, adapter-ligated sample fragment population; in step G.4, the P4 primer [SEQ ID NO: 6] is substituted for the P2 primer [SEQ ID NO:4] and the following cycling program is used for amplification using the P4 primer: 1 minute at 95.degree. C. and 3 minutes at 74.9.degree. C.

DEPR:
The digestion mixture is incubated at 37.degree. C. for 2 to 4 hours. Digestion products (500 ng), along with 500 ng aliquots of undigested difference product; amplified, adapter-ligated control fragment population; and amplified, adapter-ligated sample fragment population are analyzed on a 2.0% Seakem.RTM. GTG.RTM. (FMC BioProducts, Rockland, Me.) agarose mini-gel run in TAE buffer (40 mM Tris-acetate/10 mM ethylene-diaminetetraacetic acid, pH 8.3 at 23.degree. C.). Marker lanes include 1 .mu.g each of markers IX and III (Boehringer Mannheim, Indianapolis, Ind.).

DEPR:
The amplification products should range in size from 200-1300 base pairs. As the number of rounds of subtractive hybridization/selective amplification increases the presence of individual discrete bands should increase in intensity. If the bands are smeary reamplify with diluted sample fragments and/or decrease the number of amplification cycles. Finally, it is always preferable to use PCR in a Hotstart format.

DEPL:
The reactions are placed in a PTC 100.TM. thermocycler (M J Research, Watertown, Mass.) with Hot Bonnet and incubated for 3 minutes at 72.degree. C. (to dissociate the Linker Adapter 13-mers from step D). One microliter (5 Units)

Vent.RTM. DNA polymerase (New England Biolabs, Inc., Beverly, Mass.) is added and incubation at 72.degree. C. is continued for 5 minutes. This fills in the ends complementary to the 45-mer adapter oligonucleotide that was ligated to the fragments in step D, thereby generating a set of primer binding sites. Next, the reaction mixtures are subjected to twenty cycles of 1 minute at 95.degree. C., 30 seconds at 58.4.degree. C., and 3 minutes at 72.degree. C. A final extension of 10 minutes at 72.degree. C. is performed, then the reactions are cooled to 4.degree. C. It is important not to increase the cycle number beyond 20, as a skewing toward smaller amplification products will occur, which will bias the subsequent subtraction. If more product is required, the number of reactions should be increased.

DETL:

AO: 45-mer adapter oligonucleotide: 5'-CGATAGTCAC TCTACCACTC AGCCTACGCA CGAGACGATG TACTC-3' SEQ ID NO:1 LA: 13-mer linker-adapter: 5'-GATCGAGTAC ATC-3' SEQ ID NO:2 P1: 22-mer oligonucleotide primer (T.sub.m = 58.4.degree. C.) 5'-CGATAGTCAC TCTACCACTC AG-3' SEQ ID NO:3 P2: 24-mer oligonucleotide primer (T.sub.m = 61.9.degree. C.) 5'-TAGTCACTCT ACCACTCAGC CTAC-3' SEQ ID NO:4 P3: 24-mer oligonucleotide primer (T.sub.m = 72.1.degree. C.) 5'-TACCACTCAG CCTACGCACG AGAC-3' SEQ ID NO:5 P4: 26-mer oligonucleotide primer (T.sub.m = 74.9.degree. C.) 5'-CAGCCTACGC ACGAGACGAT GTACTC-3' SEQ ID NO:6 PP4: 26-mer 5'-phosphate oligonucleotide primer 5'-p-CAGCCTACGC ACGAGACGAT GTACTC-3' SEQ ID NO:7

CLPR:
7. The method according to claim 1, wherein the targeting primer contains a 5'-phosphate group and the non-targeting primer lacks a 5'-phosphate group.

CLPR:
12. The method according to claim 1, wherein said primer binding sites have different annealing temperatures.

CLPR:
13. The method according to claim 12, wherein each primer binding site, progressing from outermost to innermost, has a successively higher annealing temperature.

CLPR:
14. The method according to claim 13, wherein said oligonucleotide contains four primer binding sites.

CLPR:
21. The method according to claim 1 wherein one or more of the multiple primer binding sites overlaps one or more of the other primer binding sites.

CLPV:
(a) covalently attaching, to the polynucleotides in both populations, an oligonucleotide comprising multiple primer binding sites, said multiple primer binding sites comprising an outermost primer binding site, an innermost primer binding site, and one or more internal primer binding sites therebetween, to produce marked sample and control polynucleotide populations;

CLPV:
(b) amplifying the marked sample polynucleotide population using a non-targeting primer complementary to the outermost primer binding site of said oligonucleotide to generate an amplified sample polynucleotide population;

CLPV:
(c) amplifying the marked control polynucleotide population using a targeting primer complementary to the innermost primer binding site of said oligonucleotide to generate an amplified control polynucleotide population;

CLPV:
(e) subjecting the annealing mixture to conditions under which all polynucleotides in said mixture will be degraded, except for double-stranded polynucleotides containing a non-targeting primer in each strand, to generate a treated annealing mixture; and

CLPV:
(f) subsequently subjecting the treated annealing mixture to amplification, using a non-targeting primer complementary to one of said internal primer binding sites, to produce said difference product.

CLPV:
and further wherein, at each iteration, the treated annealing mixture in step (f) is subjected to said amplification using a non-targeting primer complementary to a primer binding site different from that used in the preceding cycle, to produce an additional difference product.

ORPL:
Nikiforov et al., "The use of phosphorothioate primers and exonuclease hydrolysis for the preparation of single-stranded PCR products and their detection by solid-phase hybridization" PCR Methods and Applications (1994) 3:285-291.

ORPL:
Saiki et al., "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase" Science (1988) 239:487-491.

ORPL:
Wieland et al., "A method for difference cloning: Gene amplification following subtractive hybridization" Proc. Natl. Acad. Sci. USA (1990) 87:2720-2724.

09/622,353

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 NEWS  3  Feb 06      Engineering Information Encompass files have new names
 NEWS  4  Feb 16      TOXLINE no longer being updated
 NEWS  5  Apr 23      Search Derwent WPINDEX by chemical structure
 NEWS  6  Apr 23      PRE-1967 REFERENCES NOW SEARCHABLE IN CAPLUS AND CA
 NEWS  7  May 07      DGENE Reload

 NEWS EXPRESS  May 23 CURRENT WINDOWS VERSION IS V6.0a,
               CURRENT MACINTOSH VERSION IS V5.0C (ENG) AND V5.0JB (JP),
               AND CURRENT DISCOVER FILE IS DATED 06 APRIL 2001
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=> s subtract?(10a)hybridiz?(10a)amplif?

L1          146 SUBTRACT?(10A) HYBRIDIZ?(10A) AMPLIF?
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=> s l1 and transposable element#

L2 1 L1 AND TRANSPOSABLE ELEMENT#

=> s l2 and terminal inverted repeat sequence#

L3 0 L2 AND TERMINAL INVERTED REPEAT SEQUENCE#

=> s l2 and adapter#

L4 0 L2 AND ADAPTER#

=> d l2 bib ab kwic

L2 ANSWER 1 OF 1 MEDLINE
AN 97449300 MEDLINE
DN 97449300 PubMed ID: 9305769
TI Early genes induced in hepatic stellate cells during wound healing.
AU Lalazar A; Wong L; Yamasaki G; Friedman S L
CS ARO Volcani Center, Bet Dagan, Israel.
NC DK26743 (NIDDK)
RO1 DK37340 (NIDDK)
SO GENE, (1997 Aug 22) 195 (2) 235-43.
Journal code: FOP; 7706761. ISSN: 0378-1119.
CY Netherlands
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199710
ED Entered STN: 19971224
Last Updated on STN: 19990129
Entered Medline: 19971027
AB Activation of mesenchymal cells is a central event in the wound healing response of most tissues. In liver, the mesenchymal element responsible for organ fibrosis is the hepatic stellate cell (HSC) (formerly known as lipocyte or Ito cell). The phenotypic cascade of stellate cell activation in liver fibrosis has been well documented and involves both marked morphologic changes and upregulation of several functional components including extracellular matrix, cytokine receptors, contractile filaments and metalloproteinases. However, the genetic regulation of stellate cell activation is poorly understood. In an attempt to clone genes that are involved in the regulation of HSC activation we have combined cDNA library
amplification by PCR with **subtraction**
hybridization/differential screening, and have successfully identified genes induced in vivo during early stellate cell activation in a rat model of liver fibrosis. The subtracted cDNA library comprised less than 100 unique sequences. Of these, 13 clones with sizes ranging from 322
to 745 were sequenced and characterized. Gene induction in HSCs was monitored by RNAse protection assay during early liver injury induced by the hepatotoxin CCl4. The sequenced cDNAs corresponding to the known genes
included type II transforming growth factor beta receptor, glutathione peroxidase I, transferrin and several clones encoding cellular retrotransposons, whose expression was not previously identified in non-parenchymal liver cells. In addition, one partial cDNA predicted a zinc-finger motif, suggesting a possible role of a novel transcriptional regulator. Our approach represents a valuable strategy for clarifying in vivo regulatory mechanisms of mesenchymal cell activation in wound healing.
AB . . . In an attempt to clone genes that are involved in the regulation of HSC activation we have combined cDNA library **amplification** by

PCR with **subtraction hybridization**/differential screening, and have successfully identified genes induced in vivo during early stellate cell activation in a rat model of liver. . .

CT Check Tags: Animal; Male; Support, U.S. Gov't, P.H.S.
Cells, Cultured
Cloning, Molecular: MT, methods
DNA Transposable Elements: GE, genetics
*Gene Expression Regulation
Gene Library
Glutathione Peroxidase: GE, genetics
Liver: CY, cytology
Liver: ME, metabolism

. . .

CN 0 (DNA **Transposable Elements**); 0 (RNA, Messenger); 0 (Receptors, Transforming Growth Factor beta); EC 1.11.1.9 (Glutathione Peroxidase)

=> s l1 and maize

L5 2 L1 AND MAIZE

=> s l5 and adapter#

L6 0 L5 AND ADAPTER#

=> s l5 and transpos? element#

L7 0 L5 AND TRANSPOS? ELEMENT#

=> s l5 and terminal inverted repeat

L8 0 L5 AND TERMINAL INVERTED REPEAT

=> d l5 1-2 bib ab kwic

L5 ANSWER 1 OF 2 BIOSIS COPYRIGHT 2001 BIOSIS
AN 1998:471909 BIOSIS
DN PREV199800471909
TI **Maize** DNA enrichment by representational difference analysis in a **maize** chromosome addition line of oat.
AU Chen, Z. Jeffrey (1); Phillips, Ronald L.; Rines, Howard W.
CS (1) Dep. Biol., Campus Box 1137, One Brookings Drive, Washington Univ., St. Louis, MO 63130 USA
SO Theoretical and Applied Genetics, (Aug., 1998) Vol. 97, No. 3, pp. 337-344.
ISSN: 0040-5752.
DT Article
LA English
AB The recent recovery of **maize** (Zea mays L.) single-chromosome addition lines of oat (Avena sativa L.) from oat x **maize** crosses has provided novel source materials for the potential isolation of **maize** chromosome-specific sequences for use in genetic mapping and gene cloning. We report here the application of a technique, known as representational difference analysis (RDA), to selectively isolate **maize** sequences from a **maize** chromosome-3 addition line of oat. DNA fragments from the addition line and from the oat parent were prepared using BamHI digestion and primer ligation followed by PCR **amplification**. A **subtractive hybridization** technique using an excess of the oat parental DNA was employed to reduce the availability for amplification of DNA fragments from the addition lines that were in common with the ones from the oat parental line. After three rounds of hybridization and amplification, the resulting DNA fragments were cloned into a plasmid vector. A DNA library containing 400

clones was constructed by this method. In a test of 18 clones selected at random from this library, four (22%) detected **maize**-specific repetitive DNA sequences and nine (50%) showed strong hybridization to genomic DNA of **maize** but weak hybridization to genomic DNA of oat. Among these latter nine clones, three detected low-copy DNA sequences and two of them detected DNA sequences specific to chromosome 3 of **maize**, the chromosome retained in the source **maize** addition line of oat. The other eight out of the 13 clones that had strong hybridization to **maize** DNA detected repetitive DNA sequences or high-copy number sequences present on most or all **maize** chromosomes. We estimate that the **maize** DNA sequences were enriched from about 1.8% to over 72% of the total DNA by this procedure. Most of the isolated DNA fragments detected multiple or repeated DNA sequences in **maize**, indicating that the major part of the **maize** genome consists of repetitive DNA sequences that do not cross-hybridize to oat genomic sequences.

TI **Maize** DNA enrichment by representational difference analysis in a **maize** chromosome addition line of oat.

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ORGN Super Taxa
Gramineae: Monocotyledones, Angiospermae, Spermatophyta, Plantae

ORGN Organism Name
Avena-sativa [oat] (Gramineae); Zea-mays [**maize**] (Gramineae)

ORGN Organism Superterms
Angiosperms; Monocots; Plants; Spermatophytes; Vascular Plants

L5 ANSWER 2 OF 2 CAPLUS COPYRIGHT 2001 ACS
AN 1997:155053 CAPLUS
DN 126:153663
TI PCR-based cDNA subtractive cloning method
IN Wang, Xun; Duvick, Jonathan P.; Briggs, Steven P.
PA Pioneer Hi-Bred International, Inc., USA
SO PCT Int. Appl., 55 pp.
CODEN: PIXXD2
DT Patent

LA English
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 9640998	A1	19961219	WO 1996-US8582	19960605
	W: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG				
	RW: KE, LS, MW, SD, SZ, UG, AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN				
	CA 2223104	AA	19961219	CA 1996-2223104	19960605
	AU 9662519	A1	19961230	AU 1996-62519	19960605
	AU 700952	B2	19990114		
	EP 832294	A1	19980401	EP 1996-921259	19960605
	R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, FI				
	US 5837468	A	19981117	US 1997-858767	19970519
	US 5853991	A	19981229	US 1997-863028	19970523
PRAI	US 1995-481687		19950607		
	WO 1996-US8582		19960605		

AB A process is described in which PCR-based cDNA libraries with anchored ends are made, a PCR-based cDNA subtracted (PCSUB) library is produced therefrom, and the ends of a cDNA clone are isolated, via PCR, from an anchored-ends library. The method uses lock-docking oligos as PCR primers: one primer (polyTV) locking over the poly(A) tail of the eukaryotic mRNA and producing the first strand synthesis, and a second primer (polyGH) that would lock onto the poly(C) tail added by terminal deoxynucleotidyltransferase to the newly synthesized strand. Thus, discrete sized PCR products result which would not necessarily require further subcloning/screening, and cDNA libraries are produced as opposed to specific cDNA clones. A PCR-based cDNA subtractive (PCSUB) library is generated by generating in the first place 2 cDNA libraries with anchored ends, one of tester DNA and one of driver DNA. The 2 libraries undergo **subtractive hybridization** and **amplification**.

In addn. to using dephosphorylated adaptors which prevent amplification of driver DNA, a biotin-tagged derived library is prepd. by use of biotin-labeled dCTP during PCR. This allows phys. sepn. (using streptavidin-coated beads) of driver and of driver/tester hybrid cDNA from the desired and amplified target cDNA, thus enhanced relative amplification of target cDNA. Having a way to remove the driver cDNA also allows for use of a higher ratio of drive/tester cDNA< and therefore more stringent subtraction of cDNA sequences which are not unique to the target cDNA. The PCSUB method results in a library representing differentially expressed mRNAs. Further, PCR and sequence information derived from cDNA clones from the PCSUB library can be utilized to screen the cDNA anchored end library for the ends of specific cDNA sequences. This approach would employ primers that are complementary to internal sequences, in conjunction with polyTV or polyGH or equiv. primers which comprise restriction enzyme recognition sequences at their resp. 5'-ends, to fish out from the library the ends of specific mRNAs. The PCSUB method was applied to isolate and analyze **maize** embryo genes that are induced by infection with the fungus Fusarium moniliforme. Three novel cDNA clones (C-11-3, G-4-5, and G-12-3) were obtained to exemplify genes that are activated upon F. moniliforme infection of germinating **maize** embryos.

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